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Published in:
Applied Microbiology and Biotechnology

DOI:
10.1007/s00253-016-8031-5

Publication date:
2017

Penicillin V acylases from Gram-negative bacteria degrade *N*-acylhomoserine lactones and attenuate virulence in *Pseudomonas aeruginosa*

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Running head: Penicillin V acylases as quorum quenching agents

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Abstract:

Virulence pathways in Gram-negative pathogenic bacteria are regulated by quorum-sensing mechanisms, through the production and sensing of *N*-acylhomoserine lactone (AHL) signal molecules. Enzymatic degradation of AHLs leading to attenuation of virulence (quorum quenching) could pave the way for the development of new antibacterials. Penicillin V acylases (PVAs) belong to the Ntn hydrolase superfamily, together with AHL acylases. PVAs are exploited widely in the pharmaceutical industry, but their role in the natural physiology of their native microbes is not clearly understood. This report details the characterization of AHL degradation activity by homotetrameric PVAs from two Gram-negative plant pathogenic bacteria, *Pectobacterium atrosepticum* (*Pa*PVA) and *Agrobacterium tumefaciens* (*At*PVA). Both the PVAs exhibited substrate specificity for degrading long chain AHLs. Exogenous addition of these enzymes into *Pseudomonas aeruginosa* greatly diminished the production of elastase and pyocyanin, biofilm formation and increased the survival rate in an insect model of acute infection. Subtle structural differences in the PVA active site that regulate specificity for acyl chain length have been characterized, which could reflect the evolution of AHL-degrading acylases in relation to the environment of the bacteria that produce them and also provide strategies for enzyme engineering. The potential for using these enzymes as therapeutic agents in clinical applications and a few ideas about their possible significance in microbial physiology have also been discussed.

Keywords:

Penicillin V acylase, *N*-acylhomoserine lactone acylase, Ntn hydrolase, quorum quenching, pathogenesis

Introduction:

Penicillin acylases are microbial enzymes that cleave the amide bond of natural penicillins (Arroyo et al. 2003), finding industrial application in the manufacture of the pharmaceutical intermediate 6-aminopenicillanic acid (6-APA). Penicillin acylases can show substrate preference for benzyl penicillin (Pen G, PGAs) or phenoxymethyl penicillin (Pen V, PVAs). Although both enzymes belong to the Ntn hydrolase superfamily (Oinonen and Rouvinen 2000), they differ in their catalytic *N*-terminal nucleophile residue (PGA-ser, PVA-cys) and their subunit composition. While PGAs are heterodimeric enzymes, PVAs are homotetramers and are evolutionarily related to bile salt hydrolases (BSHs) that deconjugate bile salts in the mammalian gut (Kumar et al. 2006) forming the cholylglycine hydrolase (CGH) group. A recent study (Panigrahi et al. 2014) has explored the phylogenetic clustering of CGHs from Gram-positive and Gram-negative bacteria into two different groups.

Quorum sensing (QS) allows the bacteria to perceive their population density (Rutherford and Bassler 2012) through the secretion of auto-inducer signal molecules and modulate gene expression to trigger specific metabolic pathways. QS has been linked to bioluminescence, bacterial virulence and swarming motility among other physiological processes (Li and Nair 2012). Bacterial pathogens including *Pseudomonas aeruginosa*, *Vibrio cholerae* and *Acinetobacter baumanii* use QS to regulate virulence genes and formation of biofilms, thereby increasing their persistence (Li and Tian 2012). Gram-negative proteobacteria use autoinducers *N*-acylhomoserine lactones (AHLs) (Churchill and Chen 2011), with a homoserine lactone ring linked via an amide bond to an acyl side chain (C_4-C_{18}) which may be saturated or unsaturated, or with a hydroxy, oxo or no substituent on the carbon at the 3-position of the *N*-linked acyl chain. Synthesized AHLs diffuse into neighbouring cells, where they modulate gene expression through binding to the LuxR family of regulators. While *Pectobacterium carotovorum* and *Agrobacterium tumefaciens* produce 3-oxo-C_6 and 3-oxo-C_8-HSLs, respectively (Uroz et al. 2009), *P. aeruginosa* utilizes C_4 and 3-oxo-C_{12}-HSLs as signals for auto-induction. Bacteria in mixed-species communities have also been known to respond to structurally related non-cognate AHLs produced by other bacteria (Winson et al. 1998).

The disruption of AHL-directed signaling (termed “quorum quenching”, QQ) through inhibition or enzymatic degradation is an attractive strategy for controlling bacterial pathogenesis and

biofilm formation (Dong et al. 2007). Enzymes that degrade AHL include lactonases (ring cleavage) and acylases (amide bond cleavage), which have been characterized from a variety of bacteria. An exhaustive list has been provided by Grandclément et al. (2016). Penicillin acylases are known to share similar structural fold and mechanistic features with AHL acylases, and the probability of substrate cross-reactivity has been suggested earlier (Kreszlak et al. 2007). Although recent studies have demonstrated activity of *Kluyvera citrophila* PGA (Mukherji et al. 2014) and aliphatic penicillin acylase from *Streptomyces lavendulae* (Torres-Bacete et al. 2015) on AHLs, both these enzymes are ser-Ntn hydrolases with heterodimeric structure. A new AHL acylase from *P. aeruginosa* (HacB) (Wahjudi et al. 2011) cleaves Pen V to a small extent; however, AHL degradation by PVA enzymes or any other cys-Ntn hydrolase has not been explored in detail so far. Moreover, the role of PVAs in microbial physiology is not been clearly understood till date, but a few possible links to quorum sensing and pathogenesis have been suggested (Avinash et al. 2016b).

In earlier reports, we have characterized the unique biochemical (Avinash et al. 2015) and structural (Avinash et al. 2016a) features of a highly active PVA from the Gram-negative *Pectobacterium atrosepticum* (*Pa*PVA). The present study describes the characterization of PVA from another related plant pathogen *A. tumefaciens* (*At*PVA, 62% sequence identity with *Pa*PVA) and elucidates the subtle structural differences between the enzymes. Further, we report the promiscuous deacylation of AHLs by these PVAs, and explore the structural interactions involved in AHL binding. The application of PVA enzymes also led to reduction in QS-regulated biofilm formation in *P. aeruginosa* PAO1 culture and the attenuation of *P. aeruginosa* virulence in *Galleria mellonella* infection models, making them attractive options for novel QQ-based therapeutic formulations.

Materials and Methods:

Bacterial strains and plasmids:

The bacterial strains and plasmids used in this study are listed in Table 1. *E. coli* DH5a and BL21 star strains were maintained on Luria-Bertani (LB) medium supplemented on appropriate

antibiotics and cultured at 37°C. Antibiotics were added (100 μg/ml ampicillin, 35 μg/ml kanamycin or 10 μg/ml tetracycline) as required.

Preparation of *At*PVA and *Pa*PVA:

The *pva* gene from *A. tumefaciens* (GenBank GI:159185562) was cloned in pET22b vector between NdeI and XhoI restriction sites using the primers AtuF (gcttga*catatg*tgcacgcgtttcgtttatatag) and AtuR (ctgaat*ctcgag*aagcccgagaaacttgaaag), and expressed in *E. coli* BL21 star cells with a C-terminal His-tag. The enzyme was purified to homogeneity using a HIS Select Ni^{2+} affinity column (Sigma) and ENrich^{TM} 650 (BioRad) size exclusion column. The protein was dialyzed against 10 mM Tris-Cl buffer pH 7.4 containing 100 mM NaCl and 1mM DTT and stored in aliquots at -20°C. *Pa*PVA was purified from recombinant *E. coli* as described earlier (Avinash et al. 2015).

PVA enzyme activity assay

Pen V hydrolysis activity was estimated by studying the formation of Schiff's conjugate with the product 6-APA and p-dimethyl amino benzaldehyde (Shewale et al. 1987). One unit (IU) of enzyme activity was defined as the amount of enzyme producing 1 μmol 6-APA in 1 min.

Biochemical characterization of *At*PVA

The Pen V hydrolysis activity was assayed at different pH (4-9) and temperatures (20-70°C) to ascertain the optimum conditions. *At*PVA stability was studied by incubating the protein in 10 mM Tris-Cl buffer pH 7.4 for 2 h at different temperatures between 30-90°C, and assaying for PVA activity at 45°C after different time intervals. Effect of pH on enzyme stability was studied by incubating the protein in 100 mM buffers of different pH (1-11) for 4 h at 25°C and assaying the residual activity. Kinetic parameters were determined by assaying the enzyme activity with increasing concentrations (5-240 mM) of penicillin V (potassium salt, Sigma) as substrate. The data were fitted using non-linear regression as detailed for *Pa*PVA earlier (Avinash et al. 2015).

***At*PVA crystallization and structure determination**

Crystallization trials were set up with *At*PVA (15 mg ml^{-1}) using the sitting drop vapour diffusion technique. The protein crystallized in the 0.1M HEPES pH 7.5 and 12% PEG 3350 condition of

the PEG Rx crystallization screen (Hampton Research, USA). The crystals were frozen in liquid nitrogen with 25% (w/v) 2, 5-hexanediol as cryoprotectant. Diffraction data were collected at 2.8 Å resolution at the SSRL-BL12-2 beamline at the Stanford Synchrotron Light Source (USA). Investigation and scaling of the diffraction data was performed using XDS (Kabsch 2010) and SCALA (Evans 2006). The *At*PVA structural model was built using molecular replacement on Phaser ver. 2.5.6 (McCoy et al. 2007) and Autobuild (Phenix), with the refined structure of *Pa*PVA (PDB ID: 4WL2) as the template. Further model building and refinement was done using Coot and Refmac5 (CCP4 software suite) respectively. *At*PVA crystallized in $P2_12_12_1$ space group with a single tetramer per asymmetric unit (Table S1, Online Resource 1).

Bioluminescence assay for detection of AHL degradation

AHL degradation activity was monitored by employing biosensors that exhibit luminescence in the presence of specific AHLs (Winson et al. 1998). Quenching of luminescence levels can be used as an indication of AHL hydrolysis by the acylase enzymes (Steindler and Venturi 2007).

0.5 µL of 5 mM AHL stock solution in DMSO was spotted to a flat-bottom µClear white microplate (Greiner Bio-One) and dissolved in 50 µL reaction mixture containing 5 µg enzyme in 100 mM NaCl, 1 mM DTT and 25 mM Tris HCl buffer pH 7.4 (for *At*PVA) or 20 mM sodium acetate buffer pH 5.2 (for *Pa*PVA). After 4 h incubation at 25°C, the enzyme was heat inactivated (80°C for 15 min), and an equal volume of modified PBS (137 mM NaCl, 2.7 mM KCl, 100 mM Na_2HPO_4, 1.8 mM KH_2PO_4) was added to each well, followed by 100 µl of 1:100 diluted overnight biosensor. Luminescence of the biosensors was measured at 30°C during a 12 h time-course using FLUOstar Omega (BMG Labtech) as described previously (Papaioannou et al. 2009). Control reactions were performed in the same manner using heat-inactivated enzyme. *E. coli* (pSB536) was used to analyze C_4-HSL degradation, *E. coli* (pSB401) for C_6- to C_8-HSL (Swift et al. 1997), and *E. coli* (pSB1075) for C_{10}- to (3-OH- and 3-oxo-) C_{12}-HSL (Winson et al. 1998). To determine the enzyme activity on AHLs, the ratio of luminescence unit to biosensor growth in OD_{600} (relative luminescence unit, RLU) from active enzymes was compared to those from inactive enzymes.

HPLC analysis

To confirm the deacylase activity of PVA enzyme on long chain AHLs, the reaction with C_{10}-HSL was analyzed by HPLC (Uroz et al. 2008). The enzymes (25 µg in 3ml of same buffer as the bioluminescence assay) were incubated with 0.4 mM C_{10}-HSL for 4 h at 25°C (heat-inactivated enzyme was used as a control). Samples of 750 µl from time 0 and 4 h were processed for detection of residual substrate, HSL and decanoic acid (Wahjudi et al. 2011).

For detection of the substrate, residual C_{10}-HSL in the reaction mixture was extracted twice with an equal volume of acidified ethyl acetate. The free HSL released during the reaction was dansylated with an equal volume of 2.5 mg ml^{-1} dansyl chloride (in acetone) and incubated overnight at 37°C (Lin et al. 2003). After SpeedVac evaporation, the sample was neutralized with 50 µl of 0.2 M HCl and diluted with acetonitrile. Decanoic acid in the sample was extracted thrice with an equal volume of hexane followed by drying under a nitrogen stream and derivatization with 4-bromomethoxy-7-methyl coumarin (BrMMC) reagent was performed as described previously (Wolf and Korf 1990).

HPLC was carried out in a Shimadzu LC-10AT VP system using a Phenomenex Luna C18 reverse-phase column (250 x 4.60 mm, 5 µm) coupled with a SPD-M10AVP PDA detector. The column was washed with 5% acetonitrile in water (solvent A), and the sample was eluted in a linear gradient to 100% acetonitrile (solvent B). C_{10}-HSL was detected at 219 nm, dansylated HSL at 267 nm, and BrMMC-derivatized decanoic acid at 328 nm (Uroz et al. 2008). Reaction control of reference substrate and products showed that the dansylation and BrMMC derivatization was specific to HSL and decanoic acid, respectively (data not shown).

Kinetics of AHL degradation by PVAs

The kinetic behavior of *At*PVA and *Pa*PVA on 3-oxo-C_{12}-HSL was determined by an end-point assay using ortho-phthalaldehyde (OPA) derivatization of the HSL product. Eight different concentrations of 3-oxo-C_{12}-HSL in which the substrate was completely soluble (0.01-0.25 mM) were prepared from DMSO stock. The reaction mixture consisted of 100 mM NaCl, 1 mM DTT and 25 mM sodium phosphate buffer pH 7.4 (for *At*PVA) or 20 mM sodium acetate buffer pH 5.2 (for *Pa*PVA). The DMSO concentration was kept at 0.8% for each reaction. Enzyme (2 µg *At*PVA or 0.5 µg *Pa*PVA) was added into the 1 ml reaction mixture; a 90 µL sample was taken

immediately and thereafter regularly at 1 min intervals. The enzyme was inactivated with 10 µL of 1M NaOH; this step did not interfere with the subsequent derivatization. After removal of enzyme by centrifugation, 50 µL was transferred into a black Fluotrac microplate (Greiner Bio-One) and mixed with 50 µL OPA reagent (Sigma-Aldrich), followed by 20 min incubation at 25°C. Fluorescence was measured on a FLUOstar Omega, BMG Labtech with an excitation at 355 nm and emission at 460 nm. A standard curve using 0-0.25 mM HSL standard prepared in reaction mixture showed a straight line that can be fitted to the following equation: $y = 77290x + 490.5$ (R^2=0.9996). Initial velocity was limited in the range of 15% substrate conversion and calculated from the standard curve. The enzyme kinetics model was analyzed by fitting the v/[S] curves in GraphPad Prism software.

Docking of AHLs to *Pa*PVA and *At*PVA

The 3D structures of C_6-HSL, C_{10}-HSL and 3-oxo-C_{12}-HSL used in the docking study were obtained from PubChem compound database. Partial atomic charges of each ligand atom were determined from OPLS_2005 all-atom force field using *LigPrep*. Grid based ligand docking program *Glide* was used for docking these ligands in the binding site of *Pa*PVA and *At*PVA. The binding site was defined as a grid box of dimension 26x26x26 Å, centered on the Cys1 residue. Receptor grid generation was followed by ligand docking where the ligands were docked flexibly using Glide's extra precision. Free energy of binding was roughly estimated by using an empirical scoring function called GlideScore, which includes electrostatic, van der Waals interaction and other terms for rewarding or penalizing interactions that are known to influence ligand binding. All structural figures were prepared using PyMol or *CCP4MG*.

Disruption of quorum sensing in *Pseudomonas aeruginosa* PAO1 by PVAs

Purified *At*PVA (0.08 mg ml^{-1}) or *Pa*PVA (0.4 mg ml^{-1}) was added to a 1:100 diluted overnight culture of *P. aeruginosa* PAO1 in 100 ml LB. Samples were taken at 6 and 24 h post inoculation, centrifuged for 5 min and supernatant was stored at -20°C until further analysis.

(i) AHLs measurement. The levels of 3-oxo-C_{12}-HSL and C_4-HSL were measured by bioluminescence assay using biosensor *E. coli* pSB1075 and pSB536 respectively (Winson et al. 1998; Swift et al. 1997). Cell-free supernatant was filtered through a 0.2 µm pore filter, and 20

µL of the sample was mixed with 180 µL of 1:100 diluted overnight biosensor culture. Light production was monitored at 30°C for 12 h.

(ii) Elastase assay. Cell-free supernatant (100 µL) was added to 900 µL of elastase buffer (100 mM Tris HCl pH 7.5; 1 mM $CaCl_2$) containing 20 mg of Elastin Congo Red (ECR, Sigma Aldrich) (Ohman et al. 1980). After 2h at 37°C, elastase activity of the supernatant was measured as A_{495}/A_{600}.

(iii) Pyocyanin assay. **C**ell-free supernatant (5 ml) was extracted with 3 ml chloroform, and re-extracted with 1 ml of 0.2 M HCl (Essar et al. 1990). After centrifugation, the absorbance of HCl layer was measured at 520 nm. Production of pyocyanin (µg ml^{-1} culture) was calculated as $(A_{520}/A_{600}) \times 17.072$.

(iv) Biofilm formation assay. The static biofilm assay was performed in a round-bottom polystyrene 96-well plate (Greiner Bio-One) using a method by Merrit et al. (2005) with modification. 0.5 mg ml^{-1} *At*PVA or 0.66 mg ml^{-1} *Pa*PVA was added to an overnight culture of *P. aeruginosa* PAO1 (0.01OD) in M9 medium (47.7 mM $Na_2HPO_4.7H_2O$; 22 mM KH_2PO_4; 8.5 mM NaCl; 18.7 mM NH_4Cl; 2 mM $MgSO_4$; 0.1 mM $CaCl_2$; 0.01 mM glucose). A minimum of 20 wells per treatment were used with an aliquot of 110 µL in each well. Biomass quantification was performed using a crystal violet method (Chow et al. 2014) after 18 h at 30°C.

(v) Galleria mellonella killing assay. Larvae of *G. mellonella* were obtained from Frits Kuiper (Groningen, The Netherlands) and kept in a dark container at 15°C. Animals of 2.5-3 cm size were selected for the assay, with a minimum of 15 animals per treatment. An overnight culture of *P. aeruginosa* PAO1 was diluted 1:100 in LB medium, grown into an early logarithmic phase (A_{600} 0.3-0.4), and the CFU count was determined from a standard curve of CFUs/ A_{600}. The cells were then washed with sterile 10 mM $MgSO_4$, and diluted into 10^3 CFU/mL. Afterwards, 100 µL of enzyme (0.5 mg ml^{-1} *At*PVA or 0.66 mg ml^{-1} *Pa*PVA) or reaction buffer was added to 900 µL of bacteria and incubated at 30°C for 1 hour. An insulin pen (HumaPen Luxura; Lilly Nederland) was used to inject 10 µL of the culture to the last proleg of the larvae. Animals injected with 10 mM $MgSO_4$ served as a control for physical trauma. Infection development was followed for 24 hours at 30°C (Beeton et al. 2015; Koch et al. 2014b). The animals were considered dead when not reacting to touch or have turned black.

Accession code:

The structural coordinates for *At*PVA have been deposited in the PDB under the accession codes **5J9R**.

Results:

Biochemical characterization of *At*PVA

*At*PVA was expressed as a tetramer of molecular mass 148 kDa; the enzyme exhibited a specific activity of 205 $\mu molmin^{-1}mg^{-1}$with high specificity for Pen V over bile salts and other β-lactam antibiotics (Fig. S1, Online Resource 1). Maximum Pen V hydrolysis was observed at 45°C in optimum pH 6 - 7 (Fig. 1). *At*PVA was stable in the pH range 5-8, while *Pa*PVA (Avinash et al. 2015) was more stable in acidic pH (3-6). There was also a drastic reduction in *At*PVA activity and loss of tertiary structure at 60°C (Fig. 1).

*At*PVA was observed to exhibit complex kinetic behaviour similar to *Pa*PVA, showing positive cooperativity and substrate inhibition with Pen V and modulation of PVA activity in the presence of bile salts (Fig. 2a). The major difference between *At*PVA and *Pa*PVA lies in the extent of substrate inhibition; *At*PVA showed a K_i of 47.2 mM, compared to 163.1 mM for *Pa*PVA. Near complete reduction of *At*PVA activity was observed at 240 mM Pen V, while *Pa*PVA still had considerable activity (20% of V_{max}) at the same concentration (Avinash et al. 2015). Drastic reduction in Pen V hydrolysis with *At*PVA was also observed in the presence of high GDCA (glycodeoxycholate, a bile salt) concentration (Fig. 2b).

Structural analysis of *At*PVA

The structural features of *At*PVA closely resemble the *Pa*PVA structure (PDB ID 4WL2) with a few subtle differences. Although the *At*PVA tetramer (Fig. 3) possesses a similar non-planar orientation and distance between subunits as *Pa*PVA (Avinash et al. 2016a), the angle between the opposite subunits (169.6°) was closer to the planar shape of the PVA from *Bacillus sphaericus* (171°) than *Pa*PVA (158°). *At*PVA shares many similar active site residues with *Pa*PVA including the nucleophilic N-terminal cysteine (C1), and the presence of two Trp

residues (W21, W80) in the active site participating in substrate binding. Superposition of the two structures revealed that *At*PVA (and other PVAs) lack the 5-residue insertion in the loop region (61-74) near the active site in contrast to *Pa*PVA (Avinash et al. 2015). It is possible that the length of this loop might play a role in modulating the substrate inhibition in PVAs from Gram-negative bacteria. Finally, *At*PVA and *Bt*BSH (BSH from Gram-negative *Bacteroides thetaiotamicron*, PDB ID 3HBC) also lack a solvent-exposed loop covering the region 228-239 that is present in *Pa*PVA.

AHL degradation by PVAs

The ability of PVAs from Gram-negative bacteria (*Pa*PVA and *At*PVA) to hydrolyze AHL signals was evaluated to explore their possible association with quorum sensing. Incubation (4 h) of long chain AHLs with pure PVA enzymes showed reduction in bioluminescence compared to the heat-inactivated control, indicating AHL degradation. Activity of *Pa*PVA was restricted to C_{10} and C_{12}-HSL. *At*PVA was active on a broader substrate spectrum (C_6 to C_{12}-HSL), although significant quenching was observed with the long chain AHLs, with moderate activity on C_6 and C_8-HSLs (Table 2). Both enzymes were observed to be distinctly more active on straight chain AHLs, with only moderate quenching in case of oxo- or hydroxy- substituted AHLs. The activity of the PVA enzymes on long chain AHLs was further confirmed by monitoring the degradation of C_{10}-HSL using HPLC (Fig. 4).

Kinetics of AHL degradation

For kinetic analysis, 3-oxo-C_{12}-HSL was chosen as a representative substrate as it is a highly studied signal produced by *P. aeruginosa* and has significant clinical relevance (Cooley et al. 2010; Miyari et al. 2006). *Pa*PVA (18.9 $\mu molmin^{-1}mg^{-1}$) exhibited 4-fold higher activity over *At*PVA (4 $\mu molmin^{-1}mg^{-1}$) with 0.2 mM 3-oxo-C_{12}HSL as substrate, similar to the trend for Pen V as substrate (Avinash et al. 2015).

*At*PVA and *Pa*PVA showed sigmoid v/[S] curves with increasing concentrations of 3-oxo-C_{12}-HSL, exhibiting a better fit for allosteric behaviour. However, saturation could not be achieved for both the enzymes as the low solubility of 3-oxo-C_{12}-HSL in aqueous buffer did not permit rate measurements at concentrations higher than 0.25 mM. A reasonable estimate of kinetic parameters calculated by applying initial values as constraints to the allosteric sigmoidal

equation revealed similar $K_{0.5}$ values but a significantly higher V_{max} for *Pa*PVA (Fig. 5). Apparent k_{cat}/K_m values for *Pa*PVA ($13.5x10^4$ $M^{-1}s^{-1}$) and *At*PVA ($2.68x10^4$ $M^{-1}s^{-1}$) were comparable to the available value for HacB acylase ($7.8x10^4$ $M^{-1}s^{-1}$) (Wahjudi et al. 2011) and 10 fold higher than PvdQ acylase ($5.8x10^3$ $M^{-1}s^{-1}$) (Koch et al. 2014a).

Binding of long chain AHLs to *At*PVA and *Pa*PVA

Docking studies were performed to understand the structural interactions responsible for the selective activity of PVAs on long chain AHLs. The mode of binding was almost identical in both PVAs, with the AHLs (C_6-HSL, C_{10}-HSL and 3-oxo-C_{12}-HSL) binding to the active site with similar amide bond orientation and favourable binding energy (Fig. S2, Online Resource 1). However, the extent of interaction of enzyme residues with the substrate molecule seemed to increase with the increase in length of acyl chain of the AHL molecule.

The lactone ring was housed in the same pocket where the β-lactam moiety was bound in the case of Pen V (Avinash et al. 2016a) with an Asn residue (N250 in *At*PVA or N271 in *Pa*PVA) involved in hydrogen bonding with the NH group of the amide bond. The AHL acyl chain fits into a hydrophobic pocket lined primarily by the two Trp residues in the active site (W23, W87 in *At*PVA and W21, W80 in *Pa*PVA respectively) and residues from loop 2 and loop3 surrounding the active site (Fig. 6). It appears that longer hydrophobic chains in C_{10}-HSL and 3-oxo-C_{12}-HSL enable greater number of hydrophobic interactions with the enzyme. The loop residues (Y61, L137, A138 in *At*PVA and F63, M69, L146 and A147 in *Pa*PVA respectively) form additional interactions with the hydrophobic acyl chain in these substrates, probably enhancing the strength of binding and favourably orienting the AHL molecule in the active site. Better binding affinity values (estimated as glidescores) and smaller nucleophilic attack distances from the N-terminal catalytic cysteine (C1) to the carbonyl carbon of the substrate were also observed in C_{10}-HSL and 3-oxo-C_{12}-HSL over C_6-HSL (Table 3). The presence of a (oxo- or hydroxy-) substituent did not effect a significant change in binding orientation, although a reduction in activity was observed (Table 3). It is possible that a change in polarity due to the presence of a 3' substituent might have caused a binding impediment. A preference for unsubstituted AHLs has also been observed in AHL acylases from *Shewanella* sp. (Morohoshi et al. 2008) and *Acinetobacter* sp. (Ochiai et al. 2014).

Quorum quenching in *P. aeruginosa* by PVAs

Exogenous addition of the PVAs into *P. aeruginosa* PAO1 culture was followed by measurement of AHL levels and monitoring of QS-regulated virulence factors and biofilm formation, to study their quorum quenching activity. Decrease in 3-oxo-C_{12}-HSL levels was apparent 6h post incubation (Fig. 7a), but the accumulation of C_4-HSL was unaffected (data not shown). This result corroborates the finding that both PVAs hydrolyze only long chain AHLs.

Elastase and pyocyanin levels were also negatively influenced at 6 h after acylase addition (early stationary phase) (Fig. 7b). Interestingly, *At*PVA almost completely blocked the production and pyocyanin and elastolytic activity even at 5-fold lower concentration than *Pa*PVA, despite *Pa*PVA exhibiting higher activity on AHLs *in vitro*. This might be explained by a probable loss in enzyme (*Pa*PVA) activity at pH 7 required for *P. aeruginosa* growth, or proteolytic degradation of the enzymes in bacterial culture. Although there was comparable decrease in 3-oxo-C_{12}-HSL levels in both cases after 6 h, the insufficient stability of *Pa*PVA might have caused a delay in AHL degradation, giving the bacteria time to activate the QS circuit. Decrease in QQ-mediated attenuation of virulence over time has been observed earlier in the case of AhlM from *Streptomyces* sp. (Park et al. 2005).

PVA-mediated AHL degradation also led to a moderate reduction in biofilm formation by *P. aeruginosa* (Fig. 7c). Weakening of biofilm structure in *P. aeruginosa* has been linked to the disruption of the 3-oxo-C_{12}HSL regulated *lasI/R* QS system (DeKievit et al. 2001). In addition, the therapeutic effects of PVAs in attenuation of *P. aeruginosa* virulence could be ascertained by studies on *G. mellonella* larvae. Simplicity of use and a positive correlation between *P. aeruginosa* virulence patterns in insects and mice make *G. mellonella* an attractive alternative infection model for anti-virulence experiments (Papaioannau et al. 2013; Jander et al. 2000). In the present study, preincubation of *P. aeruginosa* culture (10 cfu) with PVAs was observed to increase the survival rates of *G. mellonella* larvae after 24 h from only 10.3±7.2% in untreated infection to 73±5% (*At*PVA) or 53.7±11% (*Pa*PVA) (Fig. 7d). Control injection with only $MgSO_4$ only did not affect the survival of the larvae. Here too as in the *in vitro* assay, *At*PVA turned out to be more efficient in attenuating virulence. Regardless, these results establish the potential efficacy of PVAs as QQ therapeutic agents.

Discussion:

Enzymes active on AHLs hold great potential for application as QQ agents in clinical therapy as they can reduce virulence without affecting the growth of the bacteria, thereby diminishing the chance for emergence of resistant strains. Apart from the many AHL acylases and lactonases characterized so far, it has recently come to light that other related enzymes can promiscuously degrade the AHL signals as well, effecting QQ albeit at a lower rate. Examples include mammalian paraoxanases (Dong et al. 2007), porcine acylase (Xu et al. 2003) and PGA from *Kluyvera citrophila* (*Kc*PGA) (Mukherji et al. 2014). Although PVAs and PGAs come under the same functional ambit, they show significant differences in sequence and structural composition. While AHL acylases are generally homologous to heterodimeric PGAs and share similar active sites including an N-terminal catalytic serine, bacterial PVAs are homotetrameric and evolutionarily related to BSHs with cysteine at the N-terminal. The heterodimeric acylase from *Streptomyces avendulae* (*Sl*PVA) active on aliphatic penicillins and Pen V has been recently hinted to degrade AHLs (Torres-Bacete et al. 2015), but it shares significant sequence and structural homology with the ser-Ntn hydrolases. In the present study, the ability of cys-Ntn PVAs from Gram-negative bacteria to degrade long chain AHLs and attenuate QS-mediated virulence in *P. aeruginosa* has been described for the first time. Both the organisms employed in this study are also well-known plant pathogens that produce AHLs and employed as model systems to study AHL-based QS mechanisms (Steindler and Venturi 2007). The AiiB (Liu et al. 2007) and BlcC/AttM (Carlier et al. 2003; White et al. 2009) lactonases from *A. tumefaciens* have been implicated in QQ; however, no acylase active on AHLs has been reported so far from these bacteria.

Acylases active on AHLs have been observed to vary in their substrate specificities, and separate into different phylogenetic clusters (Ochiai et al. 2014). Enzymes of the AAC group (including AAC from *Shewanella* sp., PvdQ from *P. aeruginosa*, AhlM from *Streptomyces* sp. and AiiD from *Ralstonia* sp.) degrade only long chain AHLs, while some members of the penicillin G acylase group (including QuiP and HacB from *P. aeruginosa*, and AiiC from *Anabena* sp.) group can act on both long and short chain AHLs. A newly characterized AHL acylase AmiE of the amidase family (Ochiai et al. 2014) possesses an activity preference for long chain unsubstituted

AHLs similar to PVAs. However, the PVA enzymes shared little sequence similarity (<15%) with any of the known acylases active on AHLs (Fig. S3, Online Resource 1). In addition, both the PVAs explored in this study did not act on the AHL signals secreted by the bacteria that produce these enzymes – 3-oxo-C_8-HSL of *A. tumefaciens* and 3-oxo-C_6-HSL of *P. atrosepticum*. It would be however, interesting to study whether the substrate spectrum of penicillin acylases would include the non-canonical aryl HSLs (Ahlgren et al. 2011) as well, given that penicillins also possess aryl side chains.

Docking analysis showed that the AHLs bind to PVA enzymes at the same site as Pen V, with the acyl chain housed in a hydrophobic pocket lined by Trp residues and loop 2 and 3 while the lactone ring interacts with residues from loop 4. Accommodation of the AHL acyl chains in the active site hydrophobic pocket has been illustrated in the AHL acylase PvdQ (Bokhove et al. 2010) and *Kc*PGA (Mukherji et al. 2014), while the *S. lavendulae* acylase also contains a long hydrophobic pocket to bind aliphatic penicillins that can accommodate AHLs. The size of the hydrophobic pocket and the conformational variations of a few critical residues in the binding site have been suggested to modulate the activity of different PGAs on AHLs (Chand et al. 2015). Moreover, it has been demonstrated in PvdQ that mutagenesis of two residues (Lα146W, Fβ24Y) in the active site could change the size of the hydrophobic binding pocket thus effecting a change in substrate specificity from long chain to medium chain AHLs (Koch et al. 2014a).

PVAs occur in a diverse range of bacteria and some fungi (Avinash et al. 2016b), and are usually expressed constitutively. It has been demonstrated in *V. cholerae* (Kovacikova et al. 2003) that the PVA expression is reduced during the induction of virulence genes by the AHL-based AphA/HapR QS system and expressed more at high cell densities. Moreover, long chain AHLs have been known to antagonize QS in organisms that use C_6-C_8 HSLs as signals, including *Chromobacterium violaceum* (McClean et al. 1997) and *Aeromonas hydrophila* (Swift et al. 1997). It is therefore possible that the PVAs could be employed in the environment to gain a competitive advantage in a mixed species community (Roche et al. 2004), while not interfering with the bacterium's own QS system. Further genomic and knockout analyses of PVA producing strains could help shed some light on the relevance of their QQ ability in microbial physiology. Nevertheless, the recent additions of many novel acylases to the list of AHL-degrading enzymes

seem to go hand in hand with the complexity of AHL-based signaling mechanisms in Gram-negative bacteria.

Importantly, the knowledge of AHL-hydrolysis activity of penicillin acylases adds them to the list of QQ enzymes that can be developed for clinical applications. PVA enzyme formulations could have great potential for the biocontrol of *P. aeruginosa* pulmonary infection in cystic fibrosis patients. A dry powder formulation of the enzyme could not only be directly delivered into the lungs, but also increases its shelf life (Wahjudi et al. 2011). With their broad spectrum activity, PVAs can also help attenuate virulence in *Acinetobacter baumanii* (Chow et al. 2014) and co-infections by other pathogens whose QS mechanisms are at least partly dependent on long chain AHLs. QQ enzymes have also been applied to disrupt bacterial biofilms on silicone surfaces (Ivanova et al. 2015). Sustained QQ activity can be ensured for clinical application by enhancing protein stability (via directed evolution) and the use of stabilizing excipients. It is also advantageous that many penicillin acylases have been already optimized for industrial use with methods for their production on large scale; this could help in reducing development times for their clinical application in QQ systems. However, their activity levels and specificity for AHL acyl chain length should also be studied to direct their application to specific pathogens. With the recent expansion in the volume of information about QS systems in pathogenic bacteria, the development of a battery of enzymes acting on a broad range of AHLs would definitely prove beneficial in tackling bacterial virulence. In addition to its potential clinical application, this result also encourages the further exploration of possible link between QQ and the natural role of PVAs for the bacteria.

Acknowledgements:

V.S.A. thanks the Council of Scientific and Industrial Research (CSIR), India for Senior Research Fellowship. P.D.U. thanks Beasiswa Unggulan DIKTI Indonesia and PhytoSana project for financial support. A.P. thanks Department of Biotechnology (DBT), Government of India for CREST award. The authors also thank Priyabrata Panigrahi, NCL for help with the docking analysis.

Compliance with ethical standards:

Conflict of interest: All the authors declare that they have no conflict of interest.

Ethical approval: All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

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Table 3 Properties of different AHL substrates and results of docking with *At*PVA and *Pa*PVA structures (AlogP = hydrophobicity, SA = surface area, Nadist = Nucleophilic attack distance between SH group of cys1 and carbonyl carbon atom of AHL)